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APOPTOSIS IN IPSILATERAL KIDNEY: COMPARISON BETWEEN GROUP RECEIVING VERAPAMIL AND CONTROL GROUP POST-ARTIFICAL TOTAL UNILATERAL URETERAL OBSTRUCTION

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ABSTRACT

Objective: *Obstruction of the urinary tract has marked effects on renal blood flow, glomerular filtration rate (GFR), and tubular function. Ureteral obstruction results in an injury response that can progress to irreversible renal fibrosis and tubular damage by apoptosis.* ***Material & Method:*** *Forty five rabbits aged 13-17 weeks with body weights of 1250-1750 grams were divided into 4 groups. Group 1 underwent a sham operation and group 2 had unilateral ureteral ligation to cause total obstruction. Groups 3 and 4 also underwent unilateral ureteral ligation but with verapamil given on day 0 and day 7 respectively. Apoptosis to the renal tubules were assessed after nephrectomy on day 14 using immunohistochemistry by counting the number cell deaths/high power field (hpf).* ***Results:*** *The groups that received verapamil showed significantly less apoptosis compared to those without verapamil (2,73 vs 12,46 cell deaths/hpf; p<0,05). However, there was no significant difference between groups 3 and 4 (2,73 vs 2,89 cell deaths/hpf; p>0,05), although both groups still showed more cell deaths compared to group 1 (0,38 cell deaths/hpf).* ***Conclusion:*** *Verapamil appears to significantly decrease apoptosis during total unilateral ureteral obstruction. However, it cannot replace the benefit of relieving total obstruction*

Keywords: *apoptosis, verapamil*

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INTRODUCTION

Obstructive uropathy is the obstruction of urine flow due to anatomic and functional defects of urinary tract. The prevalence of urinary tract obstruction is expected to be 3,1% of 59,064 autopsies. Ureteral obstruction can be caused by various factors and it can be classified as acute or chronic, complete or incomplete, and unilateral or bilateral.[1] Each cause has particular symptoms, generally it has similar effects on the function of the kidney, i.e., reduction of renal function, either reversible or irreversible. Long term total ureteric obstruction may cause progressive loss of nephron that results in the atrophy of medulla and cortex.[2] In acute and transient obstruction, reduction of glomerular filtration rate (GFR) and renal blood flow (RBF) is generally temporary and reversible. Rat kidneys are able to tolerate 4-7 day total obstruction before development of nephron damage. Renal function will be completely recovered if total obstruction can be eliminated before the time limit. In animal models with total obstruction of more than 4-6 weeks, only limited glomerular function can recover. Ischemia accelerates renal damage due to obstruction and limits the recovery of obstructed kidneys. Several reports wrote that glomerular filtration rate (GFR) can return to normal after obstruction for 28 days to 1,5 years. Even though there are exceptions such as these, condition of most patients with long term total obstruction may become similar to that of animal models. Only limited function is recovered after the obstruction is eliminated.[2]

Apoptosis or programmed cell death is a suicidal mechanism of cell, allowing metazoa to control the number of cells within the tissue and eliminate certain damaged or unwanted cells.[3] Studies in rats subjected to unilateral ureteral obstruction revealed an increase of cell proliferation and apoptosis in the kidney.[4] A study by Daryanto (2005),[5] total ipsilateral renal tubular cells that are apoptotic on day 14 are higher than on day 7 in rats subjected to total unilateral ureteral obstruction. Ureteral obstruction may induce pathological and functional changes in the kidney, therefore detection and early management is important to maintain kidney function before onset of irreversible kidney damage.[6]

Verapamil is a drug belonging to calcium-channel blocker (CCB), commonly used as anti-arrhythmia, anti-hypertension, and prescribed for ischemic heart disease.[7] In unilateral ureteral obstruction (UUO) vasoconstriction occurs during and after UUO. Preglomerular vasocontriction depends more on extracellular calcium than postglomerular vasoconstriction. Changes in renal vascular resistance (RVR) are due to ANG II-induced vasoconstriction from preglomerular arterioles mediated partly by voltage-gated calcium-channel. Verapamil changes preglomerular resistance through the blocking of voltage-gated calcium channel, inhibiting vasoconstriction due to the lack of calcium influx into vascular vascular muscle cells.[8] This results in vasodilatation and increased renal blood flow. This process is regarded as allowing the prevention of kidney damage. Data have shown that the apoptosis of tubular cells have an important role in tubular atrophy and renal tissue damage in unilateral ureteral obstruction. In contrast, verapamil helps to normalize apoptotic changes in the kidney. Therefore, the objective of this study was to compare renal apoptosis in UUO in those receiving and not receiving verapamil.

OBJECTIVE

To demonstrate the difference between in number of apoptotic cells of ipsilateral kidney in Oryctolagus cuniculus subjected to total unilateral ureteral obstruction with and without administration of verapamil.

MATERIAL & METHOD

This study used post test control group design, with an assumption that variable measurement was performed only after treatment and kidney removal by means of nephrectomy. Samples in this study were male rabbit (*Oryctolagus cuniculus*), aged 13-15 weeks, body weight of 1250-1750 grams, healthy and had no disability (Animal Drugs Quality and Certification Examination Board, Directorate General of Animal Husbandry, Department of Agriculture 1989). Fifty-six male rabbits were randomly allocated into 4 groups. Each group comprised of 14 rabbits. All groups were subjected to bodyweight measurement before the treatment was given. In group I, the rabbits were sham operated, and fourteen days thereafter immunohistochemical staining using apopTag was performed to identify apoptosis in the kidney after nephrectomy. In group II, the rabbits were operated by total ligation of a unilateral ureter. Fourteen days later immunohistochemical examination was performed with apopTag to identify apoptosis in ipsilateral kidney after nephrectomy. In group III, the rats were operated by total ligation of unilateral ureter and were given verapamil. Fourteen days later immunohistochemical examination was performed using apopTag for apoptosis in ipsilateral kidney after nephrectomy. In group IV, the rats were operated by ligating total unilateral ureter and were given with verapamil from day 7 to day 14. On day 14, immunohistochemical examination was performed using apopTag to observe apoptosis in ipsilateral kidney after nephrectomy.

Before operation, the rats were fasted (except drinking) for 5-6 hours. The rats were given 100 mg/kg BW ampicillin pre-operatively. Half an hour before being anesthetized, they were given atropine 1-3 mg/kg BW intramuscularly. Then, ketamine in a dose of 40 mg/kg BW was injected intramuscularly

and combined with 0.5 mg/kg BW paraldehyde to prolong the effect of anesthesia. Aseptic procedure was done at the operation field and its adjacent area using 10% povidone iodine, and then the operation field was covered with sterile cloth. A midline lower abdominal incision was made ± 5 cm, and deepened layer by layer. Thereafter, ureters were identificatied, and all groups were subjected to total ureteral ligation by ligating the ureter with 4-0 silk thread. Hemostasis was secured and surgical wound was closed.

The rabbits were cared for as appropriate and verapamil (0,5 mg/kg) was given for 14 days in UUO groups + Verapamil. On day 14 the four groups were subjected to nephrectomy and examined for apoptosis. The specimen was taken from transversal incision of rabbits' kidney, fixed with formalin and made into paraffin blocks. Immunohistochemical examination to detect apoptosis used ApopTag staining. The preparations were observed with light microscope in magnification of 400 x, and 10 visual fields (high power). Apoptotic cells showed dark colors and had picnotic nuclei. Data were statistically analyzed. Data were presented descriptively and analyzed with comparative test to compare total unilateral ureteral obstruction and total unilateral ureteral obstruction with verapamil on the proportion of apoptotic ipsilateral renal cells. The significance level was alpha = 0,05.

RESULTS

In this study supporting data and the confounding variable was the bodyweight of the experimental animals. It was expected that rabbits' bodyweight would be affect the final result of the study. To ascertain the role of that factor, a homogeneity test between groups using ANOVA test was performed.

Table 1 shows that rabbits with ligated ureter for 14 days without verapamil (total 11 rabbits) had mean bodyweight of 1590,91 ± 86,076, and in rabbits with ligated ureter for 14 days with verapamil from day 7 to day 14 (total 8 rabbits) had mean bodyweight of 1568,75 ± 96,130. In group three, rabbits with ligated ureter for 14 days with verapamil from day 0 to day 14 (total 14 rabbits) had mean bodyweight of 1610,71 ± 78,883, while in control/SHAM group (total 12 rabbits) the mean body weight was 1529,17 ± 72,169.

Table 2. Homogeneity and comparative test on the rabbits' bodyweight.

Statistical Tests	Result	Notes
Levene's Test	0,663	Homogeneous
Significance	0,580	
F Test	2,272	No significant difference
Significance	0,095	

The result in Table 2 shows data between the groups had homogeneous variance, and the result with F test revealed higher significance value than 0,05. Therefore there was no statistically significant difference in bodyweight between the treatment groups, indicating that bodyweight in this study was not a confounding variable that have influence on apoptotic cells in rabbits receiving verapamil.

Table 1. Description of the rabbit's body weight (grams).

Statistical calculation	N	Mean	Std. Deviation
Rabbits with ligated ureter 14 days without verapamil	11	1590,91	86,076
Rabbits with ligated ureter 14 days with verapamil	8	1568,75	96,130
Rabbits with ligated ureter 14 days with verapamil 0-14	14	1610,71	78,883
Control/SHAM rabbits	45	1576,67	72,169
Total	45	1576,67	85,679

Table 3 shows that apoptotic cells in group where rabbits' ureter was ligated for 14 days without verapamil had the highest mean number of cells, 12,4636 ± 12,59820, and the second highest number of apoptotic cells were found in rabbits' ureter ligated for 14 days with verapamil 7-14 with cell mean of 2,8875 ± 1,80352, and the third highest number of apoptotic cells were found in rabbits' ureter ligated for 14 days with verapamil 0-14 with cell mean of 2,7286 ± 2,10217. Control/SHAM group had apoptotic cell mean of 0,3778 ± 0,30322.

Table 4 shows that in four existing data groups, only 1 data group had normal distribution (p>0,05), while the three others had no normal distribution. One of the attempts that should be taken when the data are not normally distributed is by performing outlier test with boxplot method. From the result of boxplot test, it was found that data in group 1 that should be eliminated was 1 and 5. In group 3, data 20, 22 and 28, while in group 4 the eliminated data were 39, 40, and 43. After the abnormal data were eliminated, the results as seen in Table 5 were obtained.

Table 3. Description of apoptotic cells in ipsilateral kidney of the rabbits

Group	N	Mean	Std. Deviation
Rabbits with ligated ureter 14 days without verapamil	11	12,46	12,60
Rabbits with ligated ureter 14 days with verapamil	8	2,89	1,80
Rabbits with ligated ureter 14 days with verapamil 0-14	14	2,73	2,10
Control/SHAM rabbits	12	0,38	0,30
Total	45	4,80	7,93

Table 4. Data normality test stage 1, apoptotic cells in ipsilateral rabbit kidneys.

Group	Kolmogorov Smirnov	Sig.	Notes
Rabbits with ligated ureter 14 days without verapamil	0,360	0,001	Abnormal
Rabbits with ligated ureter 14 days with verapamil	0,170	0,200	Normal
Rabbits with ligated ureter 14 days with verapamil 0-14	0,296	0,002	Abnormal
Control/SHAM rabbits	0,379	0,001	Abnormal

Table 5. Data normality test stage 2, apoptotic cells in ipsilateral rabbit kidney.

Group	Kolmogorov Smirnov	Sig.	Notes
Rabbits with ligated ureter 14 days without verapamil	0,174	0,200	Normal
Rabbits with ligated ureter 14 days with verapamil	0,170	0,200	Normal
Rabbits with ligated ureter 14 days with verapamil 0-14	0,201	0,200	Normal
Control/SHAM rabbits	0,256	0,182	Normal

Table 6. Advanced test or post-hoc with LSD test, apoptotic cells in ipsilateral rabbit kidneys.

Group	Rabbits with ligated ureter 14 days without verapamil	Rabbits with ligated ureter 14 days with	Rabbits with ligated ureter 14 days with verapamil 0-14	Control/ SHAM rabbits
Rabbits with ligated ureter 14 days without verapamil	-	0,001*	0,001*	0,001*
Rabbits with ligated ureter 14 days with verapamil	-	-	0,87	0,001*
Rabbits with ligated ureter 14 days with verapamil 0-14	-	-	-	0,031*
Control/SHAM rabbits	-	-	-	-

Note: *significantly different

Table 7. Homogeneity and comparative test of apoptotic cells in ipsilateral rabbit kidneys.

Statistical Tests	Result	Notes
Levene's Test	7,431	Not
Significance	0,001	Homogeneous
F Test	35,543	With significant
Significance	0,001	Difference

ANOVA test revealed that variance of apoptotic cells between groups was not homogeneous ($p<0,05$). F test revealed difference in apoptotic cells between groups with significance level of 0.001. Therefore mean apoptotic cells in rabbits with ureteral ligation for 14 days without verapamil was higher than in rabbits with ureteral ligation for 14 days with verapamil 7-14. The latter was higher than that in rabbits with ureteral ligation for 14 days with verapamil 0-14, which was higher than that in control/SHAM group. Since it was ascertained that there was difference in common, it indicated that minimally there was 1 pair different groups. The subsequent assessment was the LSD test. Table 6 shows the results of this test.

Table 6 shows that the mean of apoptotic cells in rabbits with ureters ligated for 14 days without verapamil was different from those in other groups. Whereas, mean number of apoptotic cells in rabbits with ligated ureters for 14 days with verapamil for 7 - 14 days was different only from that in control group. The mean of apoptotic cells in rabbits with ligated ureter for 14 days with verapamil for 0 - 14 was different only from that in control group.

DISCUSSION

Based on Table 3 of t test results, that there was difference in apoptosis of ipsilateral kidney tubular cells obstructed for fourteen days. In obstructive uropathy there is an increase of the number of apoptotic renal tubular cells due to the presence of acute ischemia or nephrotoxin-induced renal injury.[10] Obstruction is the cause of renal failure in children and adults, which results in the loss of renal function due to apoptosis and fibrosis of renal tubule. Glomerular function changes secondary to obstructive uropathy, depending on the severity and length of obstruction. In acute ureteral obstruction, hydrostatic pressure at the proximal part of the obstruction will rise. The increase of proximal tubular pressure emerges due to the obstruction along with the increase of capsular and tubular hydrostatic pressure, which resulted in reduction of filtration pressure.[11] Change in intrarenal pressure in unilateral ureteral obstruction may result in stretched tubular cells and subsequently causes the release of mediator. Not only intrarenal pressure, other factors, such as p53 or Fas receptor, may also trigger renal apoptosis.[8]

In his study, Miyajima showed that significant renal apoptosis occurs in obstructed kidney than in non-obstructed kidney. Topcu et al., who performed ureteral obstruction in rabbits, also found increased apoptosis compared to rabbits without obstruction.[8] Similarly, Daryanto (2005) found increased renal apoptotic cells in rabbits with ligated ureters compared controls.[5] Consistent with previous findings, this study also showed the presence of significant difference in number of apoptotic cells between groups receiving unilateral ureter obstruction with verapamil, either given since the obstruction or seven day after obstruction, and that of control group. The number of apoptotic cells in unilateral ureteral obstruction with verapamil day 0 and day 7 (respectively 2,73 and 2,89) was higher than in controls, i.e., 0,38. This may indicate that in unilateral ureteral obstruction, the verapamil administration may not inhibit the occurrence of apoptosis, but only help its reduction. It can be assumed, therefore, that therapy in ureteral obstruction is relief of obstruction, while verapamil administration only serves to reduce abnormalities in kidney function during the obstruction. Data showed that apoptotic cells in rabbits with ligated ureter without verapamil had the highest mean apoptotic cells as compared to that in rabbits with ligated ureter and receiving verapamil.

Calcium antagonists is known to successfully reduce ischemia in several organs, such as heart and kidney, by decreasing blood pressure, particularly through vasodilation, and reduction of peripheral resistance to raise renal blood flow in order to maintain normal renal physiology. In this study, the number of apoptotic cells in obstructed kidney with verapamil (a calcium antagonist) is lower than the number of apoptotic cells in obstructed kidneys without verapamil.

Organic calcium-channel blockers (CCB) particularly reduce calcium influx through voltage-gated channels, resulting in reduction of calcium inflow into the cells. Schnackenberg showed that verapamil changes preglomerular resistance by inhibiting voltage-gated calcium channel. In unilateral ureteral obstruction vasoconstriction occurs during and after relief of obstruction. Preglomerular vasoconstriction depends more on extracellular calcium than on post-glomerular vasoconstriction.[8] In his study, Topcu indicated that apoptosis of renal tubular cells has an important role in occurrence of tubular atrophy and loss of kidney tissue in short-term kidney obstruction.[8] In contrast, calcium channel blockers support the reduction of apoptosis in kidney cells.

In clinical studies, verapamil provides mild protective effects against the emergence of nephrotoxicity due to cisplatin. This protective effect results from increased renal blood flow due to renal blood vessel vasodilation resulting from verapamil administration. An experimental study on rats revealed that verapamil also reduced drug-induced nephrotoxicity. Calcium channel blockers are able to inhibit calcium-mediated vasoconstriction in smooth muscle cells, therefore regarded as capable in preventing kidney damage. Similar to the study by Topcu, this study also found reduction of apoptotic cells in obstructed kidneys with verapamil administration compared to that without verapamil.

In this study the number of renal apoptotic cells was smaller in kidney obstructed with verapamil administration since day 0 than that in verapamil administration starting from day 7, although the difference was not statistically significant. This was possible because apoptotic process had occurred before the seventh day of obstruction, therefore verapamil administration on day 7 post-obstruction demonstrated a higher number apoptotic cells compared to that in verapamil administration on day 0 obstruction. However, the number of apoptotic cells in ureteral obstruction group added with verapamil administration on day 7 showed significant reduction of the number of apoptotic cells compared to ureter obstruction without verapamil. It can be assumed that verapamil administration on day 7 ureter obstruction was still better than no verapamil administration at all.

This study did not find any necrosis in ipsilateral kidney with unilateral ureteral obstruction (Figure 1). This may mean that there is no necrosis in kidneys obstructed for fourteen days. This is consistent with the theory that the primary mechanism of renal cell death in obstructive uropathy is apoptosis.[1]

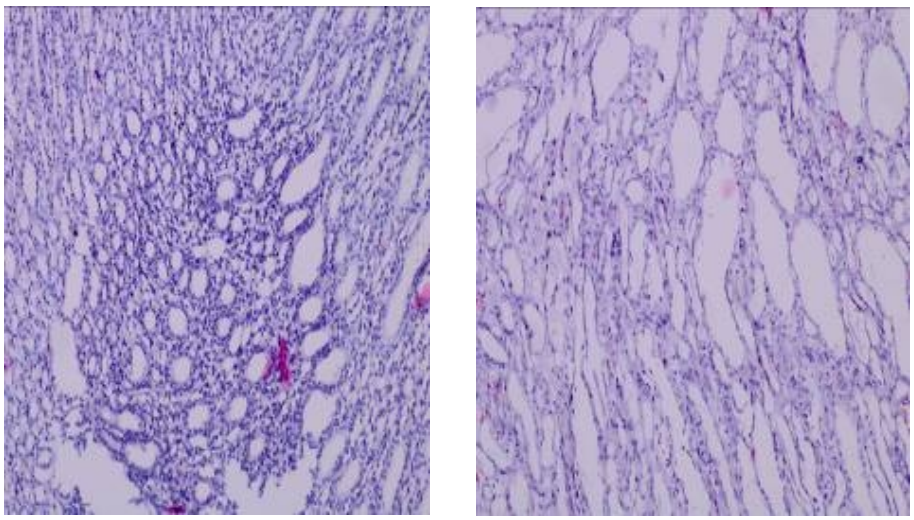

Figure 1. (A) Control group with 400x magnification, (B) Ureteral group ligated without verapamil with 400x magnification. Both figures do not show presence of necrosis.

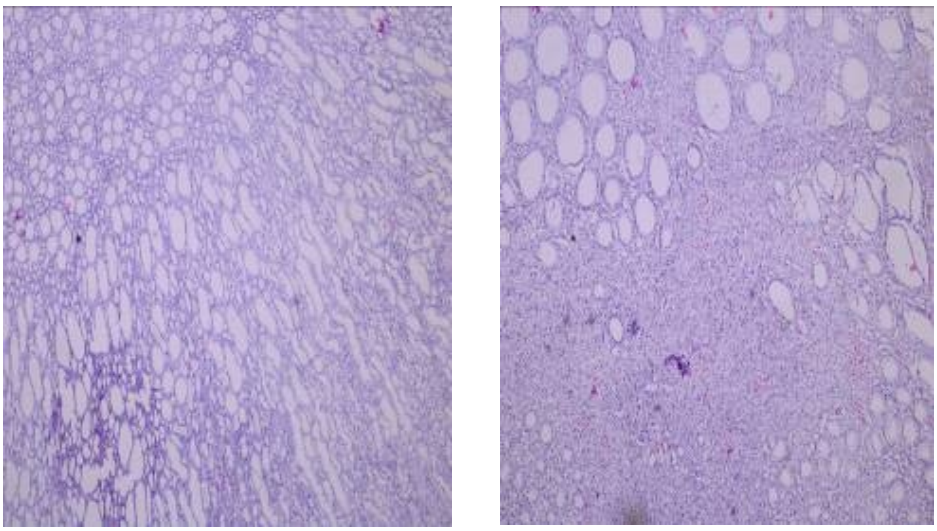

Figure 2. (C) Ligated ureter group with verapamil day 7 14 with 400x magnification, (D) ligated ureter group with verapamil day 0 14 with 400x magnification. Both figures do not indicate the presence of necrosis.

CONCLUSION

This study showed the reduction of apoptotic cells in obstructed kidney with verapamil administration compared to that without verapamil administration. Verapamil may reduce apoptosis in kidney cells during unilateral ureteral obstruction. However, the best definitive therapy for ureteral obstruction is relief of obstruction as early as possible.

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